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ORIGINAL ARTICLE

Comparison of host inflammatory responses between calcium-silicate base material and IRM

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Abstract *Background/purpose:* The use of root-end filling materials designed to stimulate tissue repair in periradicular tissues is highly recommended. These materials should be proved good tissue compatibility. The aim of this study was to estimate the responses of Intermediate restorative material (IRM), mineral trioxide aggregate (MTA), and calcium silicate (CS) cement after implantation into a rat subcutaneous, including the immune response, and materials degradation.
Materials and methods: Materials with the same chemical component were inserted into the bilateral pockets in each rat. Hematoxylin and eosin (H&E) staining was used to evaluate the immune response of tissue after implantation. Western blot was employed to quantify the COX-2 expression of tissue.
Results: After implantation for 6 weeks, H&E staining showed that the inflamed fibrous capsule surrounding MTA and CS was thicker and looser. At 12 weeks, the tissue responses became very uniform for MTA and CS. The capsule was almost free of inflammatory cells and filled with fibroblasts. A significant increase in the thickness of fibrous tissue was observed after 3 weeks, 6 weeks, and 12 weeks for IRM at the respective time points ($P < 0.05$). Significant increases of 3.45-fold, 2.81-fold, and 2.78-fold in COX-2 synthesis were observed using IRM, as compared with the control, at 3 weeks, 6 weeks, and 12 weeks, respectively ($P < 0.05$). However, CS cement was found to reduce an inflammatory reaction compared to MTA at all time points ($P < 0.05$).
Conclusion: These three cements could be considered to be biocompatible even though they induced different inflammatory responses and tissue changes during implantation tests in rats.

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CS cement did not induce acute inflammation and tissue responses similar to those reported for MTA.

Introduction

Many materials have been used for root-end fillings, such as amalgam, composite resin, glass ionomer cement, gutta-percha, mineral trioxide aggregate (MTA), and various zinc oxide- and eugenol-based cements. MTA was developed by Torabinejad et al[1] in 1993 and introduced to the market in 1998 as a root-end filling material, which is basically a mixture of 75% Portland cement, 20% bismuth oxide (Bi_2O_3), and 5% gypsum. White MTA replaced the gray one in 2002. Several studies demonstrated superior biocompatibility of MTA compared with other materials used in root-end filling and root repair; however, it has certain disadvantages such as being expensive and having poor handling characteristics.[2–4] Not only has MTA good biocompatibility, but it has also been proved to enhance hard-tissue formation.[5]

The components of MTA are similar to those of Portland cement. In a previous study, we produced a new calcium silicate (CS) cement that contained CaO, SiO_2, Al_2O_3, and ZnO. Reports have shown the physical and biological properties of CS cement to be similar to those of MTA, and CS cement is advantageous because the required setting time is shorter.[6] Additionally, CS cement not only exhibits good osteoconduction effects,[7] but also reduces inflammation of primary pulp cells.[8] Even its Si component can influence a biological response in cells.[9,10] Huang et al[11] and Shie and Ding[12] proved that extracellular regulated kinases (ERKs) were increased in cell culture. Suppression of the ERK pathway through the addition of an ERK inhibitor suggests that the effect of MTA on cells is induced by a cascade of ERK/MAPK.

Satisfactory experimental results suggest that outcomes should be compared for both *in vitro* and *in vivo* studies. Based on the findings of previous studies, this study aimed to measure the responses of intermediate restorative material (IRM), MTA, and CS cements after implantation into a rat subcutaneous, including the host tissue response, and degradation of the materials.

Materials and methods

Specimen preparation

In this experiment, IRM (Dentsply, Tulsa, OK, USA), WMTA (white ProRoot MTA; Dentsply), and CS cements were used. A detailed description of the CS powder's fabrication has been reported.[6] Appropriate amounts of as-received 65% CaO (Showa, Tokyo, Japan), 25% SiO_2 (High Pure Chemicals, Saitama, Japan), 5% Al_2O_3 (Sigma-Aldrich, St Louis, MO, USA), and 5% ZnO (Wako, Osaka, Japan) powders were mixed by a conditioning mixer (ARE-250; Thinky, Tokyo, Japan). After sintering at 1400 °C for 2 hours, the granules were ball milled in EtOH for 6 hours using a centrifugal ball mill (S 100; Retsch, Haan, Germany) and then dried in an oven at 120°C. The ground powder was mixed with 20% Bi_2O_3 (Sigma-Aldrich) and 5% $CaSO_4 \cdot 2H_2O$ (Sigma-Aldrich) using a conditioning mixer. The cement specimens were hand-mixed at a liquid-to-powder ratio of 0.35 mL/g. WMTA was used as a positive control. The liquid phase was water, and a liquid-to-powder ratio of 0.3 mL/g was used according to the manufacturer's instructions. After mixing, the cement was placed into a Teflon cylindrical mold having an internal diameter of 6 mm and thickness of 2 mm; the specimens were stored in an incubator at 100% relative humidity and 37°C for 24 hours to set. In addition, IRM (Dentsply) was used as a negative control.

Animal implantation test

The experimental protocol was approved ethically by the Animal Care Committee at the Chung Shan Medical University in Taiwan. Sixty rats weighing 180—200 g were quarantined for 2 weeks prior to the commencement of the experimental procedure. Of these, 48 rats were averagely and randomly divided into three groups, which were implanted with IRM, MTA, and CS. The remaining rats formed the control group. For animal surgery, rats were anesthetized by intramuscular injections of Zoletil 50 (25 mg/kg) and 2% Rompon (0.15 mL/kg). After the production of bilateral dorsal subcutaneous pockets, two implants with the same chemical component were inserted into the bilateral pockets in each rat. The implantation periods were 3 weeks, 6 weeks, and 12 weeks. Prior to surgery, implant materials were sterilized by immersion in 75% EtOH. Surgery was performed on the subcutaneous tissue of each animal, with the site shaved and disinfected with a 5% tincture of iodine. In the experimental group, each animal received two implants. After secure placement of the implant, the skin layer was repositioned and sutured with cotton. Control group animals received a water injection to create comparable stress. At 3 weeks, 6 week, and 12 weeks, the subcutaneous tissue was dissected and fixed in 10% formalin prior to further analysis.

Morphology

After the completion of each time period, specimens were removed from the subcutaneous tissue so that their properties could be evaluated. These specimens were washed with phosphate-buffered saline and fixed by 2% glutaraldehyde for 1 day. After dehydration in a graded ethanol series for 20 minutes at each concentration and drying at 37°C overnight, the cells were coated with a gold layer using a JFC-1600 coater (JEOL, Tokyo, Japan) and observed under a scanning electron microscope (SEM; JSM 7401F;

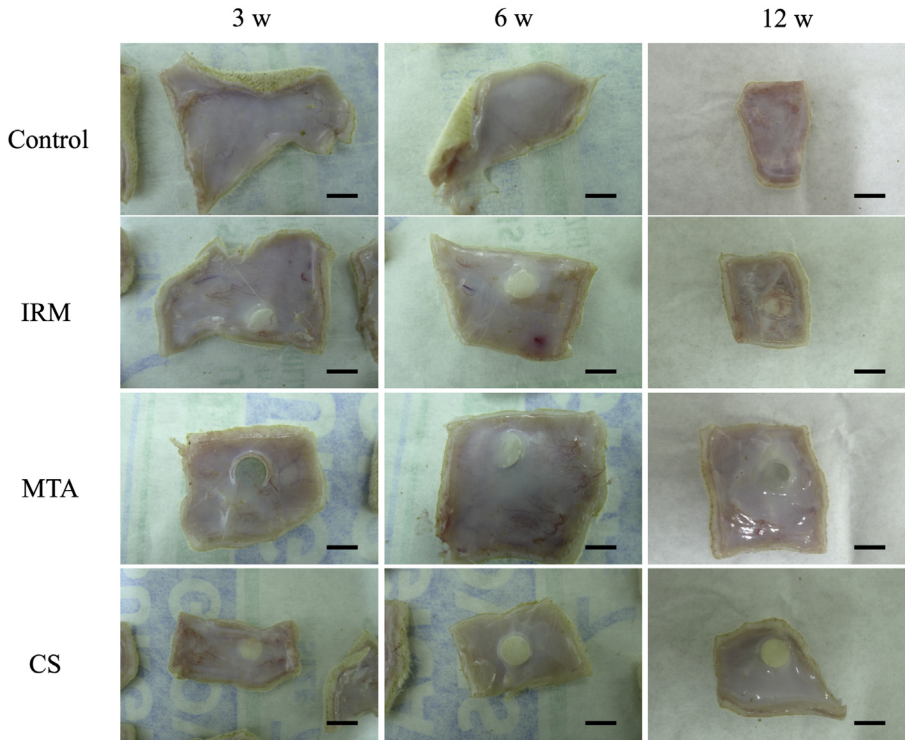

Figure 1 Photographs of different specimens implanted subcutaneously for 3, 6, and 12 weeks. The scale bar is 6 mm. CS = calcium silicate; IRM = intermediate restorative material; MTA = mineral trioxide aggregate.

JEOL) operated in the lower-secondary-electron image mode at an accelerating voltage of 3 kV.

Hematoxylin and eosin staining

The subcutaneous tissue was dehydrated using an ethanol series followed by xylene and embedded in paraffin. After embedding, the tissue was sectioned at 5 μm vertical thickness to the capsule layer using a microtome. For hematoxylin and eosin (H&E) staining, tissue sections were cleaned with pure xylene and rehydrated using a graded ethanol series. After immersion in H&E solutions for 10–20 seconds, the inflamed tissue slides were analyzed using a microscope. The average thickness of the inflamed tissue was then estimated digitally from a minimum of 15 images taken from five rats. The results were recorded as means ± standard deviation.

Western blot

The subcutaneous tissue was cut after being fixed for 2 days, washed with cold phosphate-buffered saline, and solubilized in NP-40 lysis buffer (Invitrogen, Grand Island, NY). The lysates were centrifuged at 13,000*g* for 30 minutes at 4°C. The supernatants were boiled in an sodium dodecyl sulfate (SDS) sample buffer containing 0.5 mol/L beta-mercaptoethanol. These samples were separated using SDS polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The size of the protein was determined using a protein marker (Pink Plus Prestained Protein Ladder; GeneDireX, Las Vegas, NV). After being blocked in 5% bovine serum albumin (Gibco, Langley, OK) for 1 hour, the membranes were immunoblotted with the primary antibodies COX-2 (GTX61755; GeneTex, San Antonio, TX, USA) and β-actin (GeneTex) for 2 hours. A horseradish peroxidase-conjugated secondary antibody was added subsequently, and the proteins were visualized using enhanced chemiluminescent detection kits (Invitrogen). The stained bands were scanned and quantified using a densitometer (Syngene Bioimaging System, Frederick, MD, USA) and ImageJ (National Institutes of Health, Bethesda, MD, USA). Protein expression levels were normalized to the actin band for each sample.

Statistical analysis

A one-way analysis of variance statistical test was used to evaluate the significance between the differences materials. Scheffe's multiple comparison test was used to determine the significance of the deviations in the data for each specimen. In all cases, the results were considered statistically significant at $P < 0.05$.

Results

Photographs of specimen implantation in the subcutaneous tissue

Specimens were implanted into a bilateral full skin defect in a rat model under aseptic conditions (Fig. 1). No major complications were observed during or after the operation, and no signs of complications such as infection or inflammation around the wound area were noticed. IRM and CS maintained their original colors. By contrast, MTA was initially white but changed to black after implantation.

Cell morphology

Fig. 2 shows the morphology of cells attached to IRM, MTA, and CS after implantations for 3 weeks, 6 weeks, and 12 weeks. At 3 weeks after implantation, only a few small and round cells were attached to the surface of IRM (Fig. 2A) and MTA (Fig. 2D) without spreading. By contrast, cells attached to CS surfaces were flat (Fig. 2G). After 6 weeks and 12 weeks of implantation, no increase in cell numbers was observed on IRM (Fig. 2B and C). However, SEM images showed that the cells adhered to MTA (Fig. 2E and F) and CS (Fig. 2H and I) were flat with an intact, well-defined morphology and extending filopodia, which is indicative of cellular adhesion after 6 weeks. Interestingly, several spherulites were precipitated on the cell surface after implantation for 6 weeks and 12 weeks. At high magnification (Fig. 2J), the spherulites appeared to be bone-like apatite precipitated on the cell membrane.

Inflammation tissue

Fig. 3 shows tissue response to specimen implant in the subcutaneous tissue after 3 weeks, 6 weeks, and 12 weeks, respectively. H&E staining pictures showed typical fibrous tissue formation surrounding the implants at all time points after implantation. Following implantation for 3 weeks, the tissue reaction to the specimen surface was very uneven and was characterized mainly by an inflammatory response. Six weeks after implantation, the inflamed fibrous tissue capsule surrounding MTA and CS was thicker and looser. At 12 weeks, the tissue response became very uniform for MTA and CS. The capsule was almost free of inflammatory cells and contained fibroblasts. A significant increase ($P < 0.05$) in the thickness of fibrous tissue was observed for IRM after 3 weeks, 6 weeks, and 12 weeks (Fig. 3A). To compare the different tissue responses of specimens, thickness of the fibrous tissue was evaluated (Fig. 3B). Three weeks after implantation, the fibrous tissue that formed around IRM implants (53.3 ± 2.3 μm) was significantly thicker than those formed around MTA (23.3 ± 1.5 μm) and CS (37.3 ± 3.2 μm) implants ($P < 0.05$). After implantation for 6 weeks and 12 weeks, no significant difference was observed in the thickness of the fibrous tissue that developed around the implants MTA (38.3 ± 3.2 μm and 35.0 ± 2.0 μm, respectively) and CS (43.0 ± 5.6 μm and 29.3 ± 7.4 μm, respectively). However, a significant decrease was observed in the thickness of the fibrous tissue for MTA and CS after 12 weeks ($P < 0.05$).

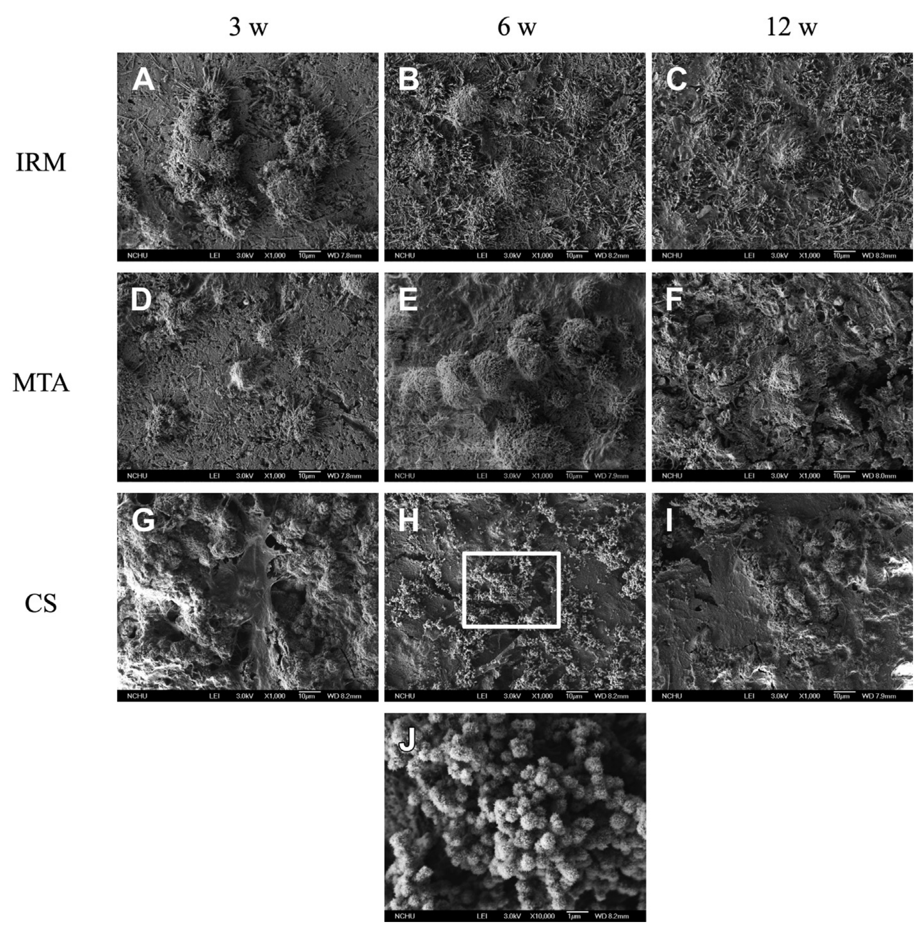

Figure 2 Scanning electron microscopy images of the surfaces of (A–C) IRM, (D–F) MTA, and (G–I) CS specimens implanted into the subcutaneous tissue for different times. (J) Image of the same surface as in (H), but with a higher magnification. CS = calcium silicate; IRM = intermediate restorative material; MTA = mineral trioxide aggregate.

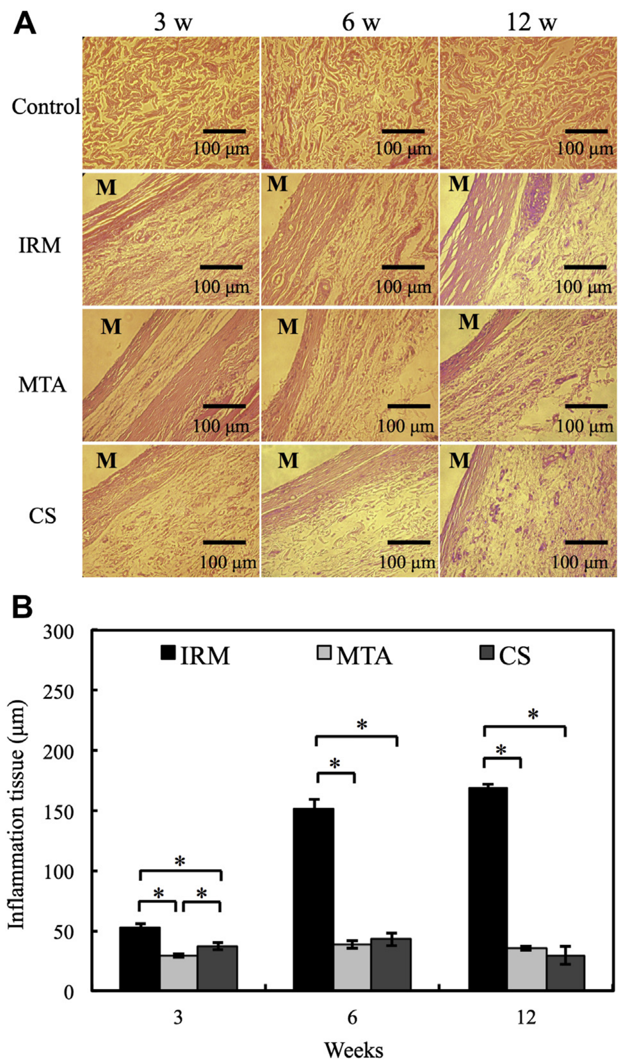

Figure 3 (A) Histological observation of different discs implanted in the subcutaneous tissue, in comparison to the control group tissues, for 3 weeks, 6 weeks, and 12 weeks (original magnification 200×, hematoxylin and eosin stain). (B) Statistical data are presented as means ± standard deviation for $n = 6$. * Significant difference ($P < 0.05$) between groups at the same time point. CS = calcium silicate; IRM = intermediate restorative material; M = material; MTA = mineral trioxide aggregate.

Western blot

To evaluate whether COX-2 proteins were stimulated when different specimens were implanted in the subcutaneous, we quantitated the activities of these proteins at various times. COX-2 production was highest in the subcutaneous after IRM implantation compared to the other groups for all time points (Fig. 4A). In all the groups, the COX-2 expression of the tissue was found to decrease with an increase in culture time (Fig. 4B). Significant increases ($P < 0.05$) of 3.45-fold, 2.81-fold, and 2.78-fold were found for COX-2 synthesis through the insertion of IRM into the subcutaneous, as compared with the control, at 3 weeks, 6 weeks, and 12 weeks, respectively. However, CS cement was found to reduce an inflammatory reaction for all implantation times when compared with MTA ($P < 0.05$). More importantly, CS indicated a decrease of approximately 23% ($P < 0.05$) in the COX-2 expression level compared to the control after 12 weeks of implantation.

Discussion

Root-end filling materials placed in contact with periodontal tissue should possess good biocompatibility. In addition, biomaterials are foreign to the host body, and the magnitude and duration of the inflammatory process have a direct impact on biocompatibility, hence affecting the efficacy of biomaterials.[13] The nonspecific (innate) immune system is responsible for initiating rapid and general responses against invasion by foreign objects. It is essential to understand the effects of CS cement on the inflammation response of tissue. In a previous study, we demonstrated that MTA and CS were calcium-silicate-based materials and both had similar physical, chemical, and biological properties.[6,7] Additionally, the viability of pulp cells cultured on CS cement was greater than the viability of those cultured on MTA for all culture times,[8] suggesting that CS cement can be used in endodontic materials.

It is known that material characteristics such as surface chemistry and roughness regulate diverse cell behavior, including cell attachment, spreading, proliferation, and differentiation.[12,14] In our SEM evaluation, the cells exhibited a rounded morphology of initial attachment within 3 weeks of IRM and MTA implantation. The change of fibroblast shape from spindle to round and the existence of blebs on the membrane surface are results of a cytoplasmic shrinkage.[15] The toxicity effect of IRM is possibly caused by free eugenol, which is strongly cytotoxic, from the cement surface as a result of progressive hydrolysis.[16] Such toxic products influence both the attachment behavior and the morphology of the cells.[9] However, the discoid-shaped cell adhered to the CS cement, which indicates a favorable interaction between the cells and the materials.[8] Moreover, after implantation in the subcutaneous tissue for 6 weeks, spherulites were found to be formed on CS surfaces. A material surface exposure to body fluids promotes the formation of a "bone-like" apatite layer, which may indicate the material's ability to integrate into tissue.[17] The bioactivity of silicate-based materials indicates that the presence of PO_4^{3-} ions in the composition is not an essential requirement for the development of an apatite layer, which consumes calcium and phosphate ions.[17] This is because PO_4^{3-} ions originate in body fluids. An increase in the pH of the environment at different time intervals was attributed to the release of $Ca(OH)_2$, which is conducive to apatite precipitation.[18,19]

Biomaterial implantation into the subcutaneous creates a wound that inevitably triggers inflammatory responses in the host. The inflammatory process affects biomaterial biocompatibility, which in turn affects the efficacy of biomedical devices.[20] In this study, the capsule layer measurement results indicated that IRM promoted growth of the fibrous capsule on the material surface *in vivo*. Statistical analysis of the inflammation response of the IRM group showed a higher level of inflammatory reaction than

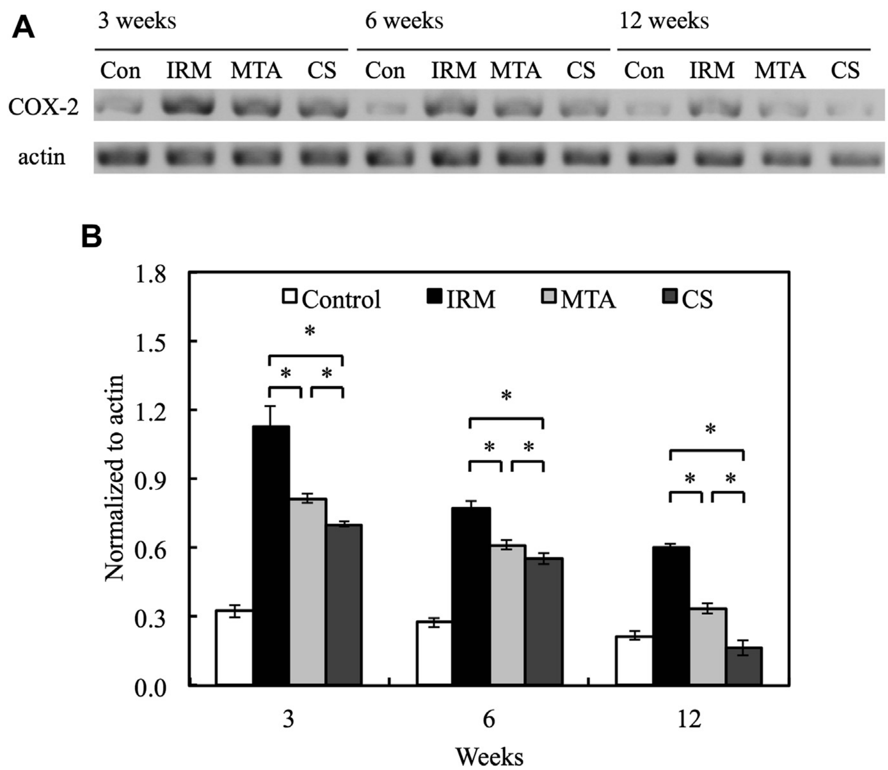

Figure 4 (A) Expression pattern of COX-2 in the subcutaneous tissue after implanting different discs for different time periods (in weeks). (B) Quantitative analyses of COX-2 immunoblotting; β-actin was used as an internal control and COX-2 level was expressed relative to the control. Statistical data are presented as means ± standard deviation for $n = 6$. * Significant difference ($P < 0.05$) between groups at the same time point. Con = control; CS = calcium silicate; IRM = intermediate restorative material; MTA = mineral trioxide aggregate.

other groups. IRM distinctly increased the inflammatory response. Histological evaluation demonstrated similar patterns of capsule formation on both MTA and CS cements. At 3 weeks after implantation, these materials showed a moderate inflammatory response. A thick fibrous capsule containing some macrophages and fibroblasts was present in the implant—tissue interface.[21] After implantation for 6 months and 12 months, the capsule became thin and dense, and was found to contain no inflammatory cells. These experiments demonstrated that the fibrous tissue capsule around MTA and CS cements is the result of a wound healing response.

A number of chemical-induced immunotoxic effects of cellular exposure to certain root-end filling materials have been reported, and several toxic compounds released from dental materials can cause an inflammatory reaction in the surrounding tissue. Although MTA and CS cements are biocompatible, they are still foreign to the tissue. During inflammation, proinflammatory cytokines typically induce the production of COX-2. Although COX-2 expression levels are normally low, in the case of various inflammatory disorders the local COX-2 expression has been shown to increase remarkably.[22] In this study, immunocompatibility was evaluated by determining the expression of COX-2 and the typical marker during cell inflammation. The COX-2 expression was low in the control group, whereas it was much higher in the MTA and CS groups, after 3 weeks of implantation ($P < 0.05$). The acute inflammatory response might have been caused by dissolution of calcium oxide in the body's fluids, probably increasing the pH around the tissue.[6] The reason for an initial inflammatory response to MTA is high pH during hydration, with the generation of inflammatory cytokines such as IL-1, IL-6, and COX-2 that contributed to the process.[23] The expression of the COX-2 marker was similar for MTA and CS.[8] Nevertheless, the COX-2 expression decreased as implantation time increased, approaching the expression of the control group after 12 weeks. The silicate-based materials caused a moderate reaction after 1 day, which decreased with time.[24] An animal study indicated that Angelus MTA upregulated adaptive immune response, but had little or no effect on pro- or anti-inflammatory cytokine production.[25]

Results showed that IRM led to acute inflammatory responses for all periods of time and was characterized by the presence of the highest COX-2 expression of fibrous tissue around the implantation material. Because IRM was a eugenol-based material, a concern has been expressed about its possible harmful effects on periapical tissues.[26] The COX-2 expression decreased as implantation time increased. In this study, no noticeable differences in the responses of fibrous tissue to either WMTA or CS cement were observed. The results may be supported by observed similarities in chemical composition and biocompatibility.[6]

In this study, IRM, MTA, and CS materials were surrounded by fibrous tissue, indicating that these were well tolerated by the tissue. The three materials investigated in this study can be assigned a biocompatibility level based on the histologic findings and the biocompatibility scale described earlier. In this study, *in vivo* degradation of these materials, as well as tissue response to them, were analyzed. These three cements can be considered to be biocompatible even though they induced different inflammatory responses and tissue changes during implantation tests in animals. The new CS cement did not induce acute inflammation, and tissue responses were very similar to those reported for WMTA. Therefore, we assume it to be suitable for endodontic use.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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